Effector-Decoy Pairs

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Effector–Decoy Pairs: Another Countermeasure Emerging during Host–Microbe Co-evolutionary Arms Races?

Plant pathogenic microbes pose a significant threat to food production, collectively affecting all cultivated crops. Given the impact of these pathogens on food security, there continues to be an urgent need to understand and exploit the biology of pathogenesis, plant susceptibility, and immunity in crop systems. Consequently, intense research efforts have helped define the molecular and evolutionary events that underpin plant-microbe interactions.

To thrive in their biotic environments, plants have acquired and evolved a robust multi-tier immune system, able to keep most would-be pathogens at bay. Microbes, able to overcome or compromise the structural and/or chemical barriers of the plant, are perceived by pattern recognition receptors (PRRs) that populate the host cell membrane. These PRRs recognize conserved microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs), and signal a microbial threat. PRR activation initiates a cellular defense response (pattern-triggered immunity [PTI]) that in most cases limits microbial ingress and prevents disease (Jones and Dangl, 2006).

Per definition, plant pathogens are adapted microbes able to infect, colonize, and reproduce on their hosts despite PTI. This suggests the presence of specialized pathogen strategies employed to inflict structural damage, promote nutrient loss, or suppress plant immunity. Genome sequencing, functional genomics, and detailed biochemical studies have firmly implicated secreted proteins (effectors) in these processes, leading to the widely held view that pathogen effectors promote host susceptibility (effector-triggered susceptibility, ETS) and enable pathogenesis (Jones and Dangl, 2006). The identification of vast candidate effector repertoires across a wide range of pathogens and pests has also prompted attempts toward their classification. In the broadest sense, effectors can be defined on the basis of their intended site of action. As their names suggest, apoplastic effectors are secreted from the pathogen cell into the host apoplast. These extracellular proteins often act as lytic enzymes (degrading structural barriers) or inhibit secreted defense-associated proteins from the host (Du et al., 2016). Cytoplasmic effectors, on the other hand, are secreted and delivered inside plant cells, binding cytoplasmic host factors in a bid to modify cellular signaling and promote susceptibility.

The identification of diverse and fast-evolving cytoplasmic effectors, and the realization that the activity of these proteins underpins both susceptibility and immunity in the presence of specific intracellular receptor-like proteins (effector-triggered immunity, ETI) has made these factors important subjects of study and agronomic application. Consequently, our understanding of cytoplasmic effector activity and recognition have driven, in large part, the establishment of conceptual models, describing host–microbe interactions whereby co-evolutionary races prompt the acquisition and evolution of sophisticated accessory systems that drive immunity and virulence in host and microbe, respectively. Importantly, recent studies in apoplastic effector–target interactions have led to important new insights, confirming and, to some degree, extending existing models (Stotz et al., 2014; Du et al., 2016).

COUNTERMEASURES IN THE HOST APOPLAST: IDENTIFICATION OF A NOVEL GLUCANASE INHIBITOR IN SOYBEAN

Recently and consistent with an important role in infection, a *Phytophthora sojae* encoded xyloglucan-specific EndoGlucanase (*Ps*XEG1) was implicated as an effector, required for infection (presumably by degrading host barriers) (Yoshizawa et al., 2012). Critically, *Ps*XEG1 also acts as a PAMP, triggering PTI responses upon recognition by an as of yet unknown PRR in its host soybean (*Glycine max*) and solanaceous species (Ma et al., 2015). These results, combined with known prevalence of secreted glucanase inhibitors in plants, prompted an immunoprecipitation-based approach to identify candidate *Ps*XEG1 inhibitors in the host. Analyses of host proteins in complex with *Ps*XEG1-EGFP resulted in the identification of soybean Glucanase Inhibitor Protein 1 (*Gm*GIP1), a protein with 37% amino acid identity to tomato-XEGIP.

Subsequent co-immunoprecipitation (CoIP), *in vitro* pull-down assays, and biochemical assays enabled the authors to establish that binding of *Gm*GIP1 to *Ps*XEG1 is strictly correlated with inhibition of xyloglucanase activity, providing strong evidence that inhibition is a direct consequence of *Gm*GIP1 binding. Given that deletion or inactivation of *Ps*XEG1 *in vivo* (by CRISPR/Cas9-mediated gene knockout and gene replacement, respectively) as well as *Gm*GIP1 overexpression impairs *P. sojae* infection, *Gm*GIP1 appears to be an important player in apoplastic immunity against *P. sojae*. It also raises the critical question as to why native levels of *Gm*GIP1 are not sufficient to limit *P. sojae* infection or indeed *Ps*XEG1 activity *in vivo* (Ma et al., 2017).

*Ps*XLP1, AN INACTIVE PARALOG OF *Ps*XEG1 IN *P. SOJAE*, CONTRIBUTES TO VIRULENCE

The inability of native levels of *Gm*GIP1 to limit *P. sojae* infection suggests the presence of pathogen-encoded co-factors that

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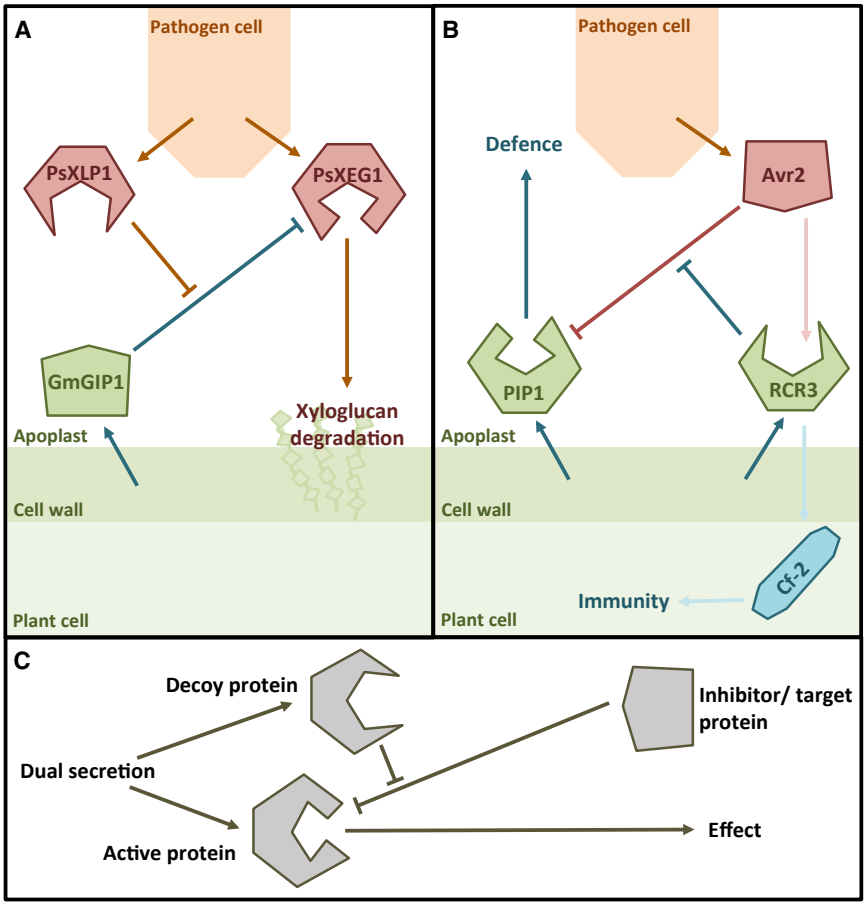

Figure 1. Schematic of the Proposed Theory of Decoy Effectors and Plant Decoy Resistance Proteins.

In both cases the cell, pathogen **(A)** or plant **(B)**, secretes two proteins, an active protein and a decoy. The decoy protein likely arises by duplication, followed by accumulation of mutations to reduce catalytic activity and to increase target affinity. The decoy then preferentially binds to and inhibits the target protein, which is intended to be an inhibitor of the active protein. The effect of the active protein is thereby restored **(C)**.

(A) The first effector–decoy pair example found expressed by a pathogen, in this case, *Phytophthora sojae*. The pathogen secretes the xyloglucan-specific EndoGlucanase 1 (*Ps*XEG1) and XEG1-Like Protein 1 (*Ps*XLP1) proteins, which are found in a head-to-head conformation within the genome and have similar expression patterns. The pair follow the general schematic with expression of the decoy *Ps*XLP1 allowing increased xyloglucan degradation *in planta*, increasing susceptibility of the plant host to infection.

(B) An example of a decoy pair found in plants, expressed by tomato on infection with the fungal pathogen *Cladosporium fulvum*. PIP1 is a protease that breaks down the proteins of invading pathogens, and Avr2 its inhibitor expressed by *C. fulvum*. In concert with PIP1 secretion the plant also secretes RCR3, thought to act as a decoy protein by binding and reducing Avr2 activity. RCR3 is also thought to activate the R gene Cf-2 upon Avr2 binding, leading to a downstream immune response.

(C) The general schematic of a decoy/active protein pair shown in **(A)** and **(B)**.

inhibit *Gm*GIP1 and thereby protect *Ps*XEG1. Since *Ps*XEG1 is a member of a protein family in *P. sojae*, the ability of *Ps*XEG1 paralogs to bind *Gm*GIP1 was tested. From these analyses one paralog (*P. sojae* XEG1-Like Protein 1 or *Ps*XLP1) was found to bind *Gm*GIP1. *Ps*XLP1 has 67% amino acid identity with *Ps*XEG1 and, importantly, has a 52-residue truncation at the C terminus that results in a loss of hydrolase activity when tested in *Nicotiana benthamiana*. Despite a loss in catalytic activity, gene expression analyses of *Ps*XIP1 in time-course infection assays revealed tight correlation with *Ps*XEG1 gene expression patterns, suggestive of a function beyond xyloglucan degradation. Indeed, mutants in which *Ps*XLP1 was disrupted or replaced were found to be severely restricted in their ability to infect host plants, whereas *Ps*XLP1 overexpression increased pathogen virulence. This indicated that despite a lack of catalytic activity toward xyloglucan, *Ps*XLP1 contributes to virulence (Ma et al., 2017).

COUNTER-COUNTERMEASURES REVEALED: *P. SOJAE* DEPLOYS *Ps*XLP1 AS A *Ps*XEG1 DECOY

To understand the mechanism by which *Ps*XLP1 contributes to virulence, two *Ps*XLP1 mutants were generated. In *Ps*XLP1^{E136A}, a mutation in the remaining theoretical active site was introduced (E to A), whereas in *Ps*XLP1^{X1,2,3} three theorized *Gm*GIP1 contact sites were mutated (regions X1, X2, and X3). CoIP of both mutants showed that *Ps*XLP1^{X1,2,3} binds very weakly to *Gm*GIP1 whereas *Ps*XLP1^{E136A} binds as strongly as wild-type *Ps*XLP1. Overexpression of the mutants demonstrated correlation between *Ps*XLP1's ability to bind *Gm*GIP1 and enhance virulence. This suggests that *Ps*XLP1 exerts its virulence function by binding to the inhibitor of *Ps*XEG1. These results invoke a model in which *Ps*XLP1 binding to *Gm*GIP1 protects catalytically active *Ps*XEG1 from being inactivated.

To test this model, competition assays were performed in which *Ps*XEG1 binding to *Gm*GIP1 was assessed in the presence or absence of *Ps*XLP1. CoIP of *Ps*XEG1 showed that *Ps*XLP1 displaces *Ps*XEG1. Critically, subsequent measurements of dissociation constants (K_D) revealed that *Ps*XLP1 bound more tightly to *Gm*GIP1 than to *Ps*XEG1. These results suggest that *Ps*XLP1 is required for *Ps*XEG1 activity, by acting on the host inhibitor *Gm*GIP1. Importantly, transgenic soybean plants overexpressing *Ps*XLP1 did not boost virulence of *Ps*XEG1-defective mutants, showing that *Ps*XLP1 binding to *Gm*GIP1 is solely intended to protect *Ps*XEG1 activity and does not contribute to virulence independent of *Ps*XEG1. Indeed, *in planta* experiments showed that both *Ps*XEG1 and *Ps*XLP1 are required to increase apoplastic sugar levels (indicative of *Ps*XEG1 activity) during infection. These results thus invoke a virulence strategy that relies on the simultaneous secretion of an effector–decoy pair during infection (Ma et al., 2017).

IMPLICATIONS AND PERSPECTIVES

It is widely accepted that both plants and their pathogens can be engaged in a co-evolutionary arms race in which molecular innovations are made. Perhaps, therefore, it is not surprising that pathogens have adopted counter-defense strategies that resemble those found in plants. It is well known that plants deploy decoys in their bid to counter pathogen infection (Figure 1). A good example is the Avr2, RCR3, PIP1 interaction (Shabab et al., 2008). AVR2 is a protease inhibitor secreted by the plant pathogen *Cladosporium fulvum* to inhibit the action of the protease PIP1. During infection, AVR2 is secreted into the apoplast where it binds PIP1 and RCR3. Although RCR3 does not act to capture or inactivate AVR2, it acts as a decoy by triggering Cf2-dependent cell death and ETI. Importantly and in contrast to the *Ps*XEG1 and *Ps*XLP1 model, RCR3 and PIP1 do not appear to be co-dependent. Although gene duplication and subsequent divergence can lead to decoy strategies in plant and pathogen that are largely analogous (Van Der Hoorn and Kamoun, 2008), important differences remain on both a mechanistic and functional level. Nonetheless, gene duplication and diversification are emerging as key events that could lead to the evolution of effector–decoy pairs. The discovery of truncated transcription activator like effectors (TALEs) in the plant pathogen *Xanthomonas oryzae*, able to suppress TALE-induced ETI (Ji et al., 2016; Read et al., 2016), is a pertinent example of this phenomenon in plant pathogens. Thus, deployment of seemingly inactive pseudogenes to protect key effector activities and undermine the host immune system represents a new virulence strategy to achieve ETS in host plants. The presence of large and complex effector gene families undoubtedly points to analogous and as yet undefined molecular innovations that are likely to emerge in the future.

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